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## Targeting matriptase in breast cancer abrogates tumor progression via impairment of stromal-epithelial growth factor signaling

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### Abstract

Matriptase is an epithelia-specific membrane-anchored serine protease that has received considerable attention in recent years due to its consistent dysregulation in human epithelial tumors, including breast cancer. Mice with reduced levels of matriptase display a significant delay in oncogene-induced mammary tumor formation and blunted tumor growth. The abated tumor growth is associated with a decrease in cancer cell proliferation. Here we demonstrate by genetic deletion and silencing that the proliferation impairment in matriptase deficient breast cancer cells is caused by their inability to initiate activation of the c-Met signaling pathway in response to fibroblast-secreted pro-HGF. Similarly, inhibition of matriptase catalytic activity using a selective

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**Author contributions:** GLZ and KL conceived the idea of the study and planned the experiments. GLZ performed the majority of the experiments and cohort maintenance. LMT, FAV and ASM performed analysis of whole tissue extracts. FAV, ASM and CB assisted with maintaining mouse cohorts. EC, RL and EM generated and characterized the IN-1 inhibitor. JL and AAM provided clinical and histopathology expertise. AAM performed the BrdU staining. JB assisted with designing experiments to study HGF mediated c-Met signaling. GLZ and KL wrote the manuscript and prepared the figures. All authors contributed to interpretation and discussion of the results and read and approved the final version.

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small-molecule inhibitor abrogates the activation of c-Met, Gab1 and AKT, in response to pro-HGF, which functionally leads to attenuated proliferation in breast carcinoma cells. We conclude that matriptase is critically involved in breast cancer progression and represents a potential therapeutic target in breast cancer.

## INTRODUCTION

Pericellular proteases in cancer progression were previously considered to be primarily extracellular matrix protein degrading enzymes. While it is clear that proteases are involved in degradation events related to breaching the basement membrane and reorganization of the extracellular matrix during invasive growth, a more complex view of pericellular proteolysis has emerged in recent years. One concept in protease mechanistic research is that proteolytic modifications of targets, including activation of growth factors, are critically involved in carcinogenesis through activation of oncogenic signaling pathways. Importantly, tumor progression is characterized by a complex interplay between invading tumor cells and stromal cells which includes paracrine interactions where growth factors secreted by stromal cells activate signaling pathways in the cancer cells.

The type II transmembrane serine protease (TTSP), matriptase, has been implicated in breast cancer since it was first discovered in breast cancer cell lines, and is highly expressed by the malignant cells in human breast carcinomas<sup>1–5</sup>. However, it is currently not known whether matriptase plays a critical role in breast cancer progression. One factor that has slowed advances on this front has been the perinatal lethality of matriptase-null mice which has, thus far, precluded direct studies of matriptase loss-of-function in the mammary gland<sup>6–7</sup>. We circumvented this obstacle by employing a matriptase hypomorphic model with low levels of matriptase in the mammary gland. When crossed into the mouse mammary tumor virus (MMTV) Polyomavirus middle T (PymT) antigen genetic mammary tumor model, matriptase hypomorphic mice displayed a significant delay in tumor onset, as well as a decreased tumor burden and tumor multiplicity. The impaired growth was caused by a profound impairment of tumor cell proliferation.

Hepatocyte growth factor or scatter factor (HGF or SF) is a pleiotrophic, paracrine growth factor and key mediator of cell migration, proliferation, survival, motility, and morphogenesis in epithelial cells<sup>8–10</sup>. HGF is biosynthesized as a single-chain zymogen-like inactive precursor (pro-HGF) and is proteolytically processed into its two-chain mature active form. The epithelial cell receptor, c-Met, binds pro-HGF or active HGF however, only the active form elicits the c-Met signaling pathway<sup>11,12</sup>. Pro-HGF is secreted by mesenchymal cells, including fibroblasts and macrophages, in the breast. Importantly, c-Met is, like matriptase, expressed on the surface of mammary epithelial cells and breast carcinoma cells<sup>5,13</sup>. The HGF/c-Met signaling pathway is dysregulated in many cancer types, including breast cancer, and has been causally linked to breast carcinogenesis<sup>13–17</sup>. Thus, transgenic expression of HGF in mouse mammary glands causes formation of multifocal invasive tumors characterized by high proliferation rates, and transgenic expression of activated c-Met leads to development of mammary hyperplasia and malignant tumors<sup>16,17</sup>. Conversely, RNAi or antibody mediated inhibition of c-Met in a variety of

cancer cells lines including breast, colon, and multiple myeloma impairs cell proliferation and invasion, and increases chemosensitivity and radiosensitivity<sup>18–21</sup>.

Since a key post-translational regulatory mechanism of oncogenic HGF/c-Met signaling is the proteolytic activation of pro-HGF, the identification of the critical activator(s) as potential targets for therapeutic intervention in cancer is important<sup>22</sup>.

Here we demonstrate that matriptase is critically involved in mammary carcinogenesis and that the molecular mechanism through which matriptase exerts its pro-carcinogenic effects is activation of pro-HGF on the cancer cell surface, leading to initiation of the c-Met signaling pathway and elicitation of mitogenic and invasive responses in breast cancer.

## RESULTS

### Epithelial matriptase is upregulated in mammary carcinomas

Transgenic MMTV-PyMT mice are predisposed to develop multifocal mammary carcinomas with tumor progression that is very similar to that seen in human breast carcinomas<sup>23,24</sup>. In order to ensure that the mouse mammary cancer model closely mimics the observations in human breast cancer, matriptase expression in normal mammary glands and mammary tumors was characterized (Fig. 1). A knock-in mouse with a promoterless  $\beta$ -galactosidase gene inserted into the endogenous matriptase gene was used as a unique tool for precise assessment of endogenous matriptase expression in the mammary gland by X-gal staining<sup>25,26</sup>. Matriptase is exclusively expressed in the epithelial cells in normal mammary glands and in the cancer cells of MMTV-PyMT oncogene induced carcinomas with no detectable expression in the stroma (Fig. 1a). Matriptase protein is detected in normal primary cells at low levels, and the expression is increased in mammary carcinoma cells as determined by western blotting (Fig. 1b). Furthermore, matriptase is present in its proteolytically active form in mammary epithelial cells, and the level of active matriptase is significantly increased in mammary carcinoma cells in comparison to normal mammary epithelial cells, as demonstrated by gelatin zymography (Fig. 1b). Importantly, the findings that matriptase is specifically expressed in the epithelial compartment and upregulated during carcinogenesis is in accordance with previous findings in human breast carcinomas rendering this tumor model particularly well-suited to understand the role of matriptase in tumor progression in humans<sup>3,5</sup>.

### Reduced matriptase in hypomorphic mammary glands

We have previously generated matriptase hypomorphic mice ( $\text{Mat}^{\text{hypo}}$ ) harboring one *St14* null allele and one *St14* “knockdown” allele in which an engrailed-2 splice acceptor site had been inserted between coding exons 1 and 2<sup>25–27</sup>. These mice display an 80–99 percent reduction in matriptase mRNA levels in different tissues and have normal postnatal and long-term survival<sup>27</sup>. To assess the level of matriptase protein in the mammary gland of  $\text{Mat}^{\text{hypo}}$  mice western blot analysis of protein lysates of whole glands was performed (Fig. 1c). The level of matriptase protein in the mammary gland in  $\text{Mat}^{\text{hypo}}$  mice is reduced >75 percent (Fig. 1d) as compared to littermate control mice ( $\text{Mat}^+$ ). Immunohistochemical staining of mammary glands also confirmed that matriptase protein expression in the ductal

epithelium is greatly reduced in the Mat<sup>hypo</sup> mice (Fig. 1e). Importantly, the residual matriptase in the mammary gland suffices to sustain normal mammary gland development based on comparative morphological and histological assessment (Supplementary Fig. 1a). Furthermore, Mat<sup>hypo</sup> females have functional mammary epithelium since they are lactation competent, and no reduction in litter size or litter weights is observed (Supplementary Fig. 1b). Thus, this model is well-suited to study progression of breast cancer in morphologically and functionally normal mammary glands with greatly reduced levels of matriptase.

### Reduced matriptase impairs mammary carcinogenesis

To investigate the effects of low matriptase levels in the mammary gland on mammary carcinoma progression, Mat<sup>hypo</sup> and Mat<sup>+</sup> mice harboring the MMTV-PyMT oncogene were generated (referred to as PymT-Mat<sup>hypo</sup> and PymT-Mat<sup>+</sup> mice, respectively). Prospective cohorts with littermate PymT-Mat<sup>hypo</sup> and PymT-Mat<sup>+</sup> virgin female mice were established and monitored by weekly palpation to determine the appearance of the first palpable mammary mass (tumor latency). At 145 days of age, the number of glands with visible and palpable tumors was recorded (tumor multiplicity) and the area of tumors was determined by caliper measurements. Total mammary tumor burden was calculated as the total weight of all postmortem excised mammary glands in individual mice from a cohort of female littermates.

Reduced levels of matriptase in the mammary glands had significant effects on all tumorigenic parameters measured (Fig. 2). Thus, the total tumor burden was reduced by 58 percent and the tumor growth rate was reduced by 76 percent (Fig. 2a,b). Also, tumor multiplicity was reduced by 45 percent (Fig. 2d). These results demonstrate that matriptase is an important contributor to tumor growth and progression. Notably, there was a significant delay in initial formation of palpable mammary tumors, indicating that matriptase is critical for early carcinogenesis with a mean latency of palpable tumors of 84 days in PymT-Mat<sup>+</sup> mice versus 120 days in PymT-Mat<sup>hypo</sup> mice (Fig. 2c). At 90 days, 53 percent of PymT-Mat<sup>+</sup> mice had palpable tumors versus 21 percent of PymT-Mat<sup>hypo</sup> mice. In order to examine the role of matriptase in early mammary carcinogenesis in further detail, an independent cohort was established and dysplastic lesions in the inguinal mammary glands were examined postmortem by whole mount analysis at 90 days of age (Fig. 2e).

Importantly, total area measurements of lesions revealed a 75 percent reduction in the glands from PymT-Mat<sup>hypo</sup> mice as compared to PymT-Mat<sup>+</sup> (Fig. 2f). Taken together, these data strongly support a functional role for matriptase in both tumor formation and tumor growth.

### Reduced matriptase impairs proliferation in mammary tumors

The cause of mammary tumor growth attenuation in mice with low matriptase was addressed through analysis of proliferation, macrophage infiltration, and apoptosis (Fig. 3). Proliferation rates of carcinoma cells were determined at two different time points by immunohistochemical detection of bromodeoxyuridine (BrdU) or endogenous Ki67 (Fig. 3). At 127 days of age, carcinoma cell proliferation was reduced by 47 percent in PymT-Mat<sup>hypo</sup> mice as compared to PymT-Mat<sup>+</sup> mice (Fig. 3a, b). A dramatic decrease in proliferation was also observed in PymT-Mat<sup>hypo</sup> 145 day old mice with an 81 percent reduction as compared to littermate PymT-Mat<sup>+</sup> mice (Fig. 3c). No significant difference in

tumor infiltration by macrophages, as determined by F4/80 staining, was detected (Fig. 3d, f), and the percentage of apoptotic cells visualized by TUNEL staining was not significantly different (Fig. 3e, g). These data strongly suggest that matriptase-dependent carcinoma cell proliferation promotes mammary tumor growth and that the decrease in proliferative mammary carcinoma cells observed in PymT-Mat<sup>hypo</sup> mice is a major contributing factor to the abrogation of primary tumor growth.

### Genetic ablation of matriptase abolishes c-Met signaling

To better understand the pathophysiological function of matriptase, its proteolytic targets need to be identified. Of the candidate substrates for matriptase that have been identified thus far, HGF has been shown to directly promote mammary carcinogenesis and therefore, is of particular interest<sup>28,29</sup>. HGF is secreted in its inactive pro-form by stromal cells in both human and mice, including stromal mammary fibroblasts and macrophages<sup>30–32</sup>.

To date, no mammary gland genetic matriptase loss-of-function studies have been published and it is unknown whether matriptase is critical for pro-HGF activation and c-Met signaling in mammary cancer. For our studies we used MMTV-PymT-induced primary mammary carcinoma cells from conditional matriptase knock-out mice (PymT-Mat<sup>cond.ko</sup>) carrying a ubiquitously expressed inducible Cre recombinase fused to a mutated human estrogen receptor (Cre-ER) which allows for permanent inactivation of floxed matriptase alleles upon brief exposure to 4-hydroxy-tamoxifen (4-OHT)<sup>33,34</sup>. As shown in Fig. 4a, efficient matriptase ablation was induced *ex vivo* in PymT-Mat<sup>cond.ko</sup> mammary carcinoma and confirmed by western blot analysis. Exposure to serum free media conditioned with fibroblast-secreted pro-HGF<sup>35</sup> caused robust c-Met activation in PymT-Mat<sup>control</sup> cells, as determined by phosphorylated c-Met (p-c-Met) protein levels (Fig. 4b). Activation of the two down-stream cytoplasmic effector proteins, Gab1 and AKT, was also observed. In contrast, in PymT-Mat<sup>cond.ko</sup> mammary carcinoma cells, activation of c-Met, Gab1, and AKT was efficiently abrogated. Importantly, stimulation of matriptase-deficient cells with pre-cleaved two-chain active HGF did not affect phosphorylation of c-Met, Gab1, or AKT (Fig. 4b). This demonstrates that the effects of matriptase gene ablation on cell signaling is caused by the loss of matriptase-mediated proteolytic conversion of inactive pro-HGF to active HGF, and thereby, its ability to initiate the c-Met signaling pathway. Also, PymT-Mat<sup>cond.ko</sup> cells retain the ability to cleave pro-HGF and activate c-Met signaling following treatment with vehicle instead of the Cre-ER recombination-inducing agent, 4-OHT (Supplementary Fig. 2)

To assess whether the matriptase-mediated activation of the HGF/c-Met signaling pathway observed in primary mammary tumor cells is relevant in whole tumors, the activation state of HGF in tumor extracts was determined in PymT-Mat<sup>hypo</sup> and PymT-Mat<sup>+</sup> mice. HGF is detected mainly in its active form in matriptase control tumors, whereas a significant fraction (approximately 50–80 percent) of the growth factor is present in its non-cleaved, single-chain, pro-form in tumors from matriptase hypomorphic mice (Fig. 4c). To further elucidate whether the reduced levels of active HGF lead to decreased activation of the c-Met signaling pathway, the levels of p-Gab1 in whole tumor lysates were analyzed. Gab-1 is a docking protein that binds the phosphorylated c-Met receptor tyrosine kinase directly and

mediates c-Met signaling. The essential role of Gab1 for signaling by the c-Met receptor has been demonstrated *in vivo*<sup>36</sup>. A pronounced decrease in P-Gab1 expression in tumors from matriptase hypomorphic mice as compared to control mice was observed, whereas the levels of total Gab1 proteins were not affected (Fig. 4d).

Taken together, these data suggest that matriptase-mediated proteolytic conversion of pro-HGF to active HGF is critical for activation of the c-Met signaling pathway, and that decreased levels of matriptase cause abated activation of the c-Met mediated signaling and proliferation *in vivo*.

### Matriptase-mediated proliferation in human breast cancer

In order to verify the relevance of matriptase-dependent pro-HGF activation in human breast cancer, we used three different human breast cancer cell lines of diverse origin: BT20, SUM229, and HCC1937, which were confirmed to express matriptase and c-Met (Fig. 4e and Supplementary Fig. 3a,b). Matriptase was efficiently silenced in all three human breast cancer cell lines using three independent, non-overlapping synthetic RNA duplexes. Addition of pro-HGF to HCC1937 matriptase-sufficient control cells resulted in robust activation of c-Met and the downstream targets, Gab1 and Akt (Fig. 4f). In contrast, matriptase ablation led to a greatly reduced level of c-Met pathway activation rendering the phosphorylated forms of c-Met, Gab1, and Akt undetectable by western blotting. Importantly, the matriptase-silenced cells displayed unaltered activation of the c-Met pathway upon stimulation with pre-cleaved, active HGF, demonstrating that the matriptase-deficient cells have a fully functional c-Met signaling pathway, and that the impaired response to pro-HGF is due to the lack of the matriptase-mediated activation cleavage (Fig. 4f). Similar results for BT20 and SUM229 cells upon matriptase silencing were observed including efficient abrogation of the activation of c-Met, Gab1 and Akt (Supplementary Fig. 3a',b''). This indicates that the mechanism observed is not limited to one cell line and may represent a general phenomenon in human breast cancer.

Matriptase, in addition to being an efficient activator of pro-HGF, has been shown to cleave and activate the pro-form of urokinase-type plasminogen activator (pro-uPA)<sup>28,29,37,38</sup>. Furthermore, it has been demonstrated that active uPA itself can cleave and activate pro-HGF *in vitro*<sup>39-41</sup>. To test whether uPA plays a role in activation of pro-HGF in breast cancer cells, silencing of uPA was performed (Supplementary Fig. 4a). RNAi mediated uPA knock-down did not result in any discernable changes in c-Met activation upon stimulation with pro-HGF, indicating that matriptase directly cleaves and activates the growth factor in a uPA independent manner in breast cancer cells (Supplementary Fig. 4b). Also, the status of uPA activation in mammary tumors was assessed by reducing western blot analysis using whole tissue lysates of tumors from matriptase hypomorphic mice and control mice. uPA is present in both its single-chain pro-form and two-chain active form in mammary tumors and no discernible differences in the pro-uPA/active uPA ratio were observed between PymT-Mat<sup>+</sup> or PymT-Mat<sup>hypo</sup> tumors (Supplementary Fig. 4c). This suggests that matriptase is not a critical activator of pro-uPA in breast cancer in the *in vivo* model used in this study.

To assess the functional consequences of inhibiting the matriptase-mediated HGF/c-Met signaling in human breast cancer cells, we assessed proliferation in the matriptase-silenced



BT20, HCC1937 and SUM229 cell lines. Following matriptase silencing in cells with three different synthetic RNA duplexes, cells were treated with pro-HGF or active pre-cleaved HGF and proliferation during 24 h was determined (Fig 4g and Supplementary Fig. 3). As expected, there was no significant proliferation difference in matriptase-sufficient HCC1937 cells treated with either active HGF or pro-HGF. In contrast, the proliferation in matriptase-silenced cells was significantly impaired in response to pro-HGF in comparison to the response observed in HGF treated cells (Fig. 4g). Similar results were obtained with the SUM229 and BT20 cell lines (Supplementary Fig. 3a'',b''). These results demonstrate that silencing matriptase leads to decreased pro-HGF activation and subsequent c-Met signaling pathway activation, ultimately causing growth inhibition. In addition, a "gain-of-function" experiment using SUM159 cells that have lost the expression of endogenous matriptase and are non-responsive to pro-HGF was performed (Supplementary Fig. 5). In spite of the loss of matriptase activity, SUM159 cells have retained the proliferation response capability upon stimulation with two-chain active HGF. Therefore, to investigate whether re-expression of matriptase could restore the ability of these cells to respond to pro-HGF, SUM159 cells were transiently transfected with a mammalian expression vector containing human full-length matriptase. Indeed, matriptase re-expression fully restored c-Met signaling in SUM159 human breast cancer cells in response to pro-HGF. The matriptase-mediated ability to activate the c-Met signaling pathway was accompanied by a strong mitogenic response to pro-HGF (Supplementary Fig. 5c).

Taken together, by performing "loss-of-function" studies for mouse mammary carcinoma cells, mouse mammary tumors, and human cancer cells, we have demonstrated that matriptase is a major activator of pro-HGF in model systems using either primary mammary carcinoma cells or three different established breast carcinoma cell lines. This conclusion is supported by the fact that no apparent compensation by one or more proteases, upon genetic ablation or post-transcriptional silencing of matriptase, was detected. Furthermore, the "gain-of-function" study demonstrates that matriptase confers SUM159 breast cancer cells with the capability to activate pro-HGF, and thereby elicit the c-Met signaling pathway and subsequent proliferative response.

### **Matriptase-mediate invasion in human breast cancer cells**

Many cancer cell lines form spontaneous, three dimensional (3D), multicellular, tumor-like spheroids when cultured in reconstituted basement membrane (rBM) overlay culture. These spheroids often resemble solid tumors, and tumorigenic processes including invasive growth, can therefore be studied in this model.

HGF has been shown to induce invasiveness in a variety of cancer cells in 3D culture. When SUM229 cells were grown in rBM overlay culture, they spontaneously formed spheroids (Fig. 5a,b). Upon stimulation with pro-HGF for 24 h, matriptase-sufficient control cells displayed pronounced invasive growth characterized by branching, multicellular structures, and single cells with a flattened spindle-like appearance (Fig 5a'). Matriptase silencing in SUM229 spheroids (Mat KD) rendered the cells unresponsive to pro-HGF, thus, keeping the vast majority of the cells confined in compact spheroid structures (Fig. 5b' and Supplementary Fig. 6). Addition of pre-cleaved active HGF restored the ability of



matriptase-deficient cells to respond to the growth factor. These data demonstrate that matriptase, in addition to promoting breast cancer cell proliferation as described above, potentiates the invasive capacity of cancer cells suggesting that matriptase-mediated c-Met signaling is critical for several pro-tumorigenic processes.

### Correlated matriptase and c-Met expression in breast cancer

To investigate whether the functional link identified between matriptase and c-Met is likely to represent a general molecular pro-tumorigenic mechanism in human breast cancer, the expression of matriptase and c-Met in a large sample set of 153 human invasive ductal carcinoma tumor tissues was analyzed by immunohistochemistry (IHC) (Fig. 6). To ensure specific staining, two different antibodies raised against matriptase and c-Met, respectively, were used. An antibody recognizing p-c-Met was also included. The staining of invasive ductal carcinomas was strong and uniform for matriptase and c-Met in the cancer cells. Furthermore, the staining patterns observed for matriptase, c-Met, and p-c-Met were strikingly similar, consistent with co-localization of the protease and the growth factor receptor (total and phosphorylated c-Met) in breast carcinomas (Fig. 6a,c). No significant staining was observed in tumor stromal compartments. The vast majority of tumor samples, 88 percent (134/153), displayed detectable expression of both matriptase and c-Met protein (Fig. 6b). A small subset of tumors, 7 percent (11/153), displayed no detectable expression of either matriptase or c-Met, whereas 5 percent (7/153) of the breast tumors expressed matriptase, but not c-Met. In only 0.7 percent (1/153) of the tumors, c-Met, but not matriptase protein was detected. The overall comparison of groups showed a highly significant correlation between matriptase and c-Met expression in breast cancer. Importantly, co-expression of matriptase and c-Met was also consistently observed in MMTV-PyMT tumors, further validating this breast tumor model as being highly relevant for studying the matriptase/pro-HGF/c-Met signaling axis (Supplementary Fig. 7).

The finding that matriptase and c-Met are both expressed in the vast majority of the human invasive ductal carcinomas analyzed, presents the opportunity for potential therapeutic intervention in a large number of breast cancer patients.

### Targeting matriptase proteolytic activity in breast cancer

Based on our findings that matriptase is critically involved in breast cancer cell proliferation and invasion, and in tumor progression and growth, *in vivo*, this protease represents a promising target for drug development. To determine whether inhibition of matriptase proteolytic activity leads to abrogation of the c-Met signaling activation, we initially tested three matriptase inhibitors in murine and human breast cancer cell culture models. Leupeptin is a small peptide, reversible protease inhibitor with a broad spectrum of protease targets. HAI-1 and 2 are reversible, competitive, macromolecular Kunitz-type serine-protease inhibitors that have been shown to be physiological inhibitors of matriptase<sup>42–45</sup>. Leupeptin, HAI-1, and 2 efficiently inhibited c-Met activation upon pro-HGF stimulation in the three human breast cancer cell lines, BT20, HCC1937, and SUM229 (Fig. 7a, Supplementary Fig. 8a). None of the three inhibitors affected c-Met activation upon stimulation with pre-cleaved active HGF. To further investigate whether matriptase may represent a novel target for pharmaceutical intervention in breast cancer, the inhibitor of matriptase containing a

ketobenzothiazole serine trap, IN-1, was tested<sup>46,47</sup>. IN-1 inhibited c-Met activation in both primary mammary carcinoma cells and in the three human breast cancer cell lines BT20, HCC1937, and SUM229 (Fig. 7b, Supplementary Fig. 8b). Notably, IN-1 inhibited pro-HGF driven proliferation in all three human cell lines in a dose-dependent manner (Fig. 7c, Supplementary Fig. 8c). The inhibitory effect observed at 10  $\mu$ M, 1  $\mu$ M, and as low as 100 nM, was so efficient that the proliferation was abrogated to a level that was statistically indistinguishable from the level observed when no growth factor was added ( $p>0.1$ ). In sum, the development of selective and efficient inhibitors of matriptase proteolytic activity represents a potential new avenue for targeting the pro-tumorigenic c-Met signaling pathway by inhibition of the matriptase-dependent conversion of the signaling-inert single-chain, pro-HGF to a signaling-competent, two-chain HGF.

## DISCUSSION

Matriptase is an epithelial cell-surface protease that is highly expressed in human breast cancer. In this study, the specific functions of matriptase in mammary tumorigenesis were investigated by using a mouse model of human ductal mammary adenocarcinoma. Mice with low matriptase expression in the mammary epithelium displayed a significant delay in tumor onset, tumor burden, and multiplicity. Notably, a significant abrogation of tumor progression was seen in early stages of carcinogenesis. A major factor contributing to tumor growth impairment was the dramatic decrease in carcinoma cell proliferation in matriptase hypomorphic tumors. To elucidate the molecular mechanisms underlying matriptase-mediated proliferation we utilized an *ex vivo* model with primary mammary carcinoma cells, as well as 2D and 3D human breast cancer cell culture models. We demonstrate that matriptase is necessary to initiate the activation of the c-Met signaling pathway in response to fibroblast-secreted pro-HGF by cleaving and activating the growth factor. HGF is an important promoter of proliferation and invasion in cancer, and its receptor, c-Met, like matriptase, is expressed in the epithelial compartment of the breast. The inactive pro-form of HGF binds to the c-Met tyrosine kinase receptor where, upon proteolytic cleavage, it is converted to the signaling-competent active form of HGF. This HGF-mediated c-Met signaling activates multiple downstream targets, including the PI3/AKT pathway, and the c-Met docking protein, Gab1. Until now, the protease responsible for pro-HGF activation in breast cancer was unknown. Using matriptase-deficient primary mammary carcinoma cells, we demonstrate that matriptase is essential for activation of fibroblast-secreted pro-HGF and initiation of downstream c-Met signaling. The *in vivo* relevance was confirmed in tumors where the levels of activated phospho-Gab1 were decreased in tumors from matriptase hypomorphic mice. Furthermore, the amounts of active tumoral HGF were reduced in matriptase hypomorphic mice.

The findings in mouse mammary cancer were verified in human breast cancer using matriptase silencing in three different cell lines, which lead to abrogation of c-Met, Gab1, and Akt activation upon pro-HGF stimulation. Furthermore, re-expression of matriptase rescued c-Met activation in a cell line that has lost endogenous matriptase expression, and rescued the capability to respond to pro-HGF. In addition, we demonstrate that the functional consequence of abrogating matriptase is severe impairment of HGF-driven proliferation and invasion in breast cancer.

Matriptase-mediated pro-HGF activation and c-Met signaling in the breast cancer models used does not appear to be dependent on uPA. This is likely due to the fact that uPA has been shown to be at least four orders of magnitude less efficient than matriptase in activating pro-HGF<sup>38</sup>.

In addition to using genetic ablation and post-transcriptional silencing of matriptase, we also employed inhibitors of matriptase catalytic activity. The small-molecule, selective matriptase inhibitor, IN-1, efficiently abrogated c-Met activation, and blocked breast cancer cell proliferation.

These data demonstrate that matriptase-mediated proteolytic pro-HGF activation, and c-Met signaling is critical in breast cancer and that targeting of matriptase proteolytic activity efficiently inhibits breast cancer cell proliferation via a mechanism conserved in mice and humans.

Since breast cancer cells express a variety of extracellular proteases, it is somewhat surprising that none of these appear to be capable of cleaving and activating pro-HGF in either mouse primary mammary carcinoma cells, or human breast cancer cells, when matriptase expression is ablated. Hepatocyte growth factor activator (HGFA) has been reported to be expressed in multiple breast cancer cell lines and in human breast tumors<sup>48</sup>. It should be mentioned however, that despite its name, no evidence has been presented that implicates HGFA as a physiological relevant pro-HGF activator. In genetic targeting studies in mice, both HGF null and c-Met null mice display an embryonic lethal phenotype, whereas HGFA-null mice develop normally to term and have no discernable phenotype in adult mice<sup>49–51</sup>. This suggests that HGFA is dispensable as a pro-HGF activator during development and postnatal life. Studies assessing the role of HGFA in tumorigenesis *in vivo* have not been published.

Two other members of the TTSP family, hepsin and TMPRSS13, that have previously been reported as being able to cleave and activate pro-HGF *in vitro*, were readily detectable by western blot analysis in a variety of breast cancer cells lines (unpublished data), which included the HCC1937, SUM229, and BT20 lines used in this study. Since no or minimal residual pro-HGF activating activity was detected upon matriptase silencing, it is unlikely that these proteases act as efficient pro-HGF activators on the cell surface of breast cancer cells. It can be argued that acute matriptase ablation may not allow breast cancer cells time to mobilize compensatory mechanisms. Countering this possibility is the fact that the SUM159 cell line that has lost endogenous matriptase, which remains undetectable through several years and multiple passages, expresses high levels of both hepsin and TMPRSS13, yet requires transgenic re-expression of matriptase in order to respond to pro-HGF stimulation. Several possible scenarios could explain why matriptase is an efficient cellular pro-HGF activator. In *in vitro* experiments using recombinant soluble active forms of hepsin and matriptase, hepsin is approximately 10-fold less efficient than matriptase in activating pro-HGF, whereas matriptase and HGFA display similar efficiency<sup>38</sup>. However, the level of active matriptase in comparison to active hepsin or active HGFA in breast cancer cells is not known. Also, matriptase is able to efficiently cleave pro-HGF on the cell surface<sup>38</sup> which may give it an advantage over HGFA, a soluble protease, as activated HGF can be

specifically generated at its site of action. It remains to be determined whether the membrane localization of matriptase provides the protease with a kinetic advantage and whether the stem-region domains are involved in substrate recognition or interaction with c-Met. Thus, factors including activation state, membrane-anchoring, localization in cell membrane microdomains, and interaction between protease and c-Met, could favor matriptase in certain cellular microenvironments, including breast cancer. It should be mentioned that it is plausible that other proteases contribute to pro-HGF activation in whole organisms (mice and human).

Based on the data presented, further development and testing of selective matriptase inhibitors may represent a new avenue for targeted therapy in breast cancer. As demonstrated, matriptase is critically involved in the activation of c-Met dependent pro-tumorigenic processes *in vivo*, *ex vivo*, and in breast cancer cell lines. Importantly, the fact that matriptase and c-Met display strongly correlated expression patterns in human breast tumors suggests that targeting the matriptase-mediated c-Met signaling cascade has the potential for broad clinical application. One factor to consider is the potential side-effects of matriptase inhibition in patients. Based on studies in mouse genetic models, the skin and the gastrointestinal tract would likely be the most sensitive tissues where matriptase targeted drugs could cause adverse effects. However, hypomorphic mice, which have less than 1 percent residual epidermal and intestinal matriptase, display only a mild and temporary ichthyosis of the skin in very young mice, and a mild gut barrier defect with no discernible consequences for adult mouse health<sup>52,53</sup>. Furthermore, humans with a hypomorphic mutation in the *St14* gene, resulting in a matriptase mutant protein with a 1000-fold lower activity than that of wild-type matriptase, have a mild temporary ichthyosis of the skin and brittle hair that shows significant improvement with age. No gastrointestinal symptoms were reported<sup>27,53</sup>. It is therefore plausible that matriptase function in normal physiological processes in humans is predominantly critical during childhood, and that targeted inhibition in adults have few and mild adverse effects. In a recent article by the group of researchers that originally identified c-Met and characterized its biochemical properties, structure, and function in development and disease, the rationale and progress of targeting c-Met in cancer is reviewed<sup>22</sup>. The c-Met inhibitors developed thus far include c-Met antagonists, such as antibodies that bind to c-Met and compete with pro-HGF/HGF binding, and c-Met kinase inhibitors. The authors mention targeting of pro-HGF activating proteases as potential novel inhibitors of c-Met signaling and that resistance to kinase inhibitors may be circumvented by combining inhibitors with different modes of action.

In conclusion, we demonstrate that matriptase is critical for breast carcinoma progression using multiple *in vivo*, *ex vivo*, and cell culture approaches. Importantly, a mechanistic understanding of the pro-cancerous properties of matriptase has been reached by identifying matriptase as an essential activator of pro-HGF and initiator of the c-Met signaling pathway. The findings described here lay the foundation for future studies testing small-molecule matriptase inhibitors and their potential as novel targeted therapeutic drugs in breast cancer.

## METHODS

### Animals

All procedures involving live animals were performed in an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited vivarium following institutional guidelines and standard operating procedures.

### Generation of *St14* hypomorphic mammary tumor mice

Mice transgenic for the MMTV-Polyoma virus middle T oncogene (*PyVT*) in the FVB/N background (strain: *FVB/N-Tg (MMTV-PyVT)<sup>634Mul</sup>*) were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and crossed with females carrying an *St14* (matriptase) null allele (*St14<sup>+/-</sup>*)<sup>6</sup> or an *St14* knock-down/hypomorphic allele (*St14<sup>+/-</sup>hypo*)<sup>27</sup> in the C57BL/6 background. F1 *PyVT/St14<sup>+/-</sup>* male pups were then mated to *St14<sup>+/-</sup>hypo* or *ST14<sup>hypo/hypo</sup>* females to produce the study cohorts of F2 hypomorphic female mice carrying the *PyVT* transgene, *PyVT/St14<sup>-/-</sup>hypo* mice (referred to as: PymT-Mat<sup>hypo</sup> mice) and littermate control mice, *PyVT/St14<sup>+/+</sup>*, *PyVT/St14<sup>+/-</sup>hypo* or *PyVT/St14<sup>+/-</sup>* (referred to as: PymT-Mat<sup>+</sup> mice). Genotyping of *St14* wild-type, knockdown, and null alleles was performed by PCR of ear or tail biopsy DNA<sup>6,23</sup>.

### Generation of *St14* conditional knock-out mammary tumor mice

The generation of the conditional matriptase knockout allele (*St14<sup>LoxP</sup>*), as well as the *St14* null allele carrying a  $\beta$ -galactosidase expression cassette (*St14<sup>E16</sup> $\beta$ -gal*), have been described previously<sup>21,27</sup>. Interbreeding of mice carrying these *St14* alleles with mice carrying a 4-hydroxytamoxifen (4-OHT)-inducible Cre transgene ( *$\beta$ -actin-Cre-ER<sup>tm+/0</sup>* mice, Strain: (B6.Cg-Tg(Cre/Esr1)5AMC/J) Jackson Laboratories), and *FVB/N-Tg (MMTV-PyVT)<sup>634Mul</sup>* mice resulted in the generation of female (*MMTV-PyVT)<sup>634Mul</sup> $\beta$ -actin-Cre-ER<sup>tm+/0</sup>;St14<sup>LoxP/E16</sup> $\beta$ -gal* study mice (referred to as PymT-Mat<sup>Cond. KO</sup>) and their associated control littermates, (*PyVT/ $\beta$ -actin-Cre-ER<sup>tm+/0</sup>;St14<sup>LoxP/+</sup>*, *PyVT/ $\beta$ -actin-Cre-ER<sup>tm+/0</sup>;St14<sup>LoxP/E16</sup> $\beta$ -gal*, *PyVT/ $\beta$ -actin-Cre-ER<sup>0</sup>;St14<sup>LoxP/E16</sup> $\beta$ -gal*, *PyVT/ $\beta$ -actin-Cre-ER<sup>0</sup>;St14<sup>LoxP/+</sup>* (referred to as PymT-Mat<sup>+</sup>). The genotypes of all mice were determined by PCR of ear or tail biopsy DNA<sup>27,34</sup>.

### Mammary gland whole mount analysis

Mammary glands were fixed in Carnoy's Fixative and stained in Carmine Alum. The area of cancerous lesions relative to the area of the entire mammary gland was determined with Image J (NIH software).

### Immunohistochemistry analysis of mouse tissue

Tissues were fixed for 24 hours in 10% neutral-buffered zinc formalin (Z-fix, Anatech, Battle Creek, MI), processed into paraffin and sectioned. Antigens were retrieved by heating in epitope retrieval buffer-reduced pH (Bethyl Laboratories, Montgomery, TX). The sections were blocked for 1 hour at room temperature with 3% bovine serum albumin in PBS and incubated overnight at 4°C with primary antibody<sup>34</sup>. Primary antibodies used were sheep anti-matriptase (1:100) and goat anti-c-Met (1:100)(R&D Systems, Minneapolis, MN). Cell

proliferation was visualized by intraperitoneal injection of 100µg/g bromodeoxyuridine (BrdU) (Sigma Chemical Co, St. Louis, MO) 2 h before euthanasia. BrdU incorporation was detected with a rat anti-BrdU antibody (Accurate Chemical and Scientific Corporation, Westbury, NY, 1:10). Alternatively, a rabbit anti-Ki67 antibody (1:100) (Thermo Scientific, Logan, UT) was used. As negative controls, non-immune rabbit IgG, non-immune sheep IgG, or non-immune goat IgG (Sigma, St. Louis, MO) were used (adjusted to same final concentration as specific primary antibodies). Macrophages were detected by a rabbit anti-F4/80 antibody (1:100) (Abcam, Cambridge, MA). Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) was performed using a TACS® 2 TdT-DAB In Situ Apoptosis Detection Kit (Trevigen, Gaithersburg, MD) according to the manufacturer's instructions.

### Breast cancer tissue array

The use of human tissue paraffin embedded samples was approved according to the Institutional Review Board Administration. The "BC20812 and "BRC711" breast cancer tissue arrays with pathology grading were obtained from US Biomax, Inc. (Rockville, MD). Grade II and III tumors were analyzed in this study. Tissue arrays were deparaffinized with xylene and hydrated with graded ethanol solutions. Antigen retrieval was performed using heated citrate buffer, reduced pH (Bethyl Laboratories, Montgomery, TX). The arrays were blocked with 2 % bovine serum albumin in PBS, and immunostained overnight at 4°C<sup>5</sup>. Primary antibodies were mouse anti-matriptase (1:200), goat anti-c-Met (1:200) and rabbit anti-phospho-cMet (1:200) (R&D Systems, Minneapolis, MN), rabbit anti-matriptase (1:100) (Calbiochem/EMD Millipore, San Diego, CA), rabbit anti c-Met (1:30)(Leica Microsystems Inc., Buffalo Grove, IL) and rabbit anti-phospho-cMet (1:200) (Abcam, Cambridge, MA). As negative controls, non-immune mouse IgG, rabbit IgG or goat IgG were used (Sigma, St. Louis, MO) (adjusted to same final concentration as specific primary antibodies).

### Isolation of primary mammary epithelial cells

Tumor tissue was removed aseptically, minced and digested with hyaluronidase (300 U/ML) (EMD Chemicals, Gibbstown, NJ) and collagenase (2 mg/ml) (Worthington Biochemical Corporation, Lakewood, NJ) added to growth medium: Ham's F12:DMEM (Corning Cellgro, Manassas, VA) supplemented with 5% FCS (Atlanta Biologicals, Lawrenceville, GA), insulin (10 µg/ml), EGF (10 ng/ml), transferrin (10 µg/ml) (Lonza, Walkersville, Maryland) and hydrocortisone (10 S (Atlanta Biologicals, Lawrenceville, GA), ins and incubated for 2 hours at 37°C. Normal primary mammary cells were isolated from inguinal glands 4 and 9 and digested as above for 30 min at 37°C. Primary epithelial cells or carcinoma cells were then isolated by centrifugation and plated in growth medium<sup>30,54</sup>. Growth medium was supplemented with Penicillin/Streptomycin/Gentamicin (Thermo Scientific, Logan, UT).

### Human breast cancer cell lines

The SUM159 and SUM229 cell lines were a gift from Dr. Stephen Ethier (Medical University of South Carolina, Charleston, SC). The HCC1937 and BT20 breast cancer cells were purchased from ATCC (Mannassas, VA). SUM159 and SUM229 cells were grown in



5% IH media (Ham's F-12 media, supplemented with 5% FBS, 1 µg/ml hydrocortisone, and 5 µg/ml insulin), BT-20 cells were grown in Eagles + NEAA media (Eagle's MEM with 2 mM L-glutamine and Earle's BSS adjusted to contain 1.5g/L sodium bicarbonate, 0.1 mM non-essential amino acids, 1mM sodium pyruvate, and 10% FBS), SUM190 cells were grown in SFIH media (Ham's F-12 media, supplemented with 1 µg/ml hydrocortisone, and 5 µg/ml insulin, 5 mM ethanolamine, 10 mM HEPES, 5 µg/ml transferrin, 10 nM triodo-thyronine, 50 µM sodium selenite, and 5% BSA) and HCC 1937 cells were grown in RPMI + L-GLUT media (RPMI-1640 media with 2 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, 1 mM sodium pyruvate, and 10% FBS).

### **Matriptase synthetic inhibitor**

A selective, slow, tight-binding inhibitor of matriptase containing a ketobenzothiazole serine trap (IN-1) was designed based on the auto-catalytic domain (RQAR) of matriptase. Details on the synthesis and characteristics of the inhibitor have been described previously<sup>46</sup>.

### **Pro-HGF/SF activation assays**

Pro-HGF/SF was produced in the immortalized human fibroblasts cell line, RMF-HGF<sup>35,55</sup>. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing high glucose and supplemented with 10% fetal bovine serum, penicillin/streptomycin, and 2 mM L-glutamine (all Invitrogen Corporation, Carlsbad, CA). To collect the pro-HGF/SF, the cells were washed twice with sterile PBS, incubated for 8 h in serum-free DMEM, after which the medium was replaced by fresh serum-free DMEM. The conditioned medium was collected after 72 h, aliquoted and stored at -80 °C. To determine the quality and concentration of each preparation, 40 µl of the conditioned medium was loaded on 4–12% reducing SDS-PAGE and analyzed by Western blotting using a goat-anti human HGF primary antibody (R&D Systems, Minneapolis, MN, 1:1000) with human recombinant active HGF (R&D Systems, Minneapolis, MN) as a loading control. Human breast cancer cell lines or primary mouse MMTV-PyMT mammary carcinoma cells were plated in 6- well plates and grown to confluence. Cells were then serum starved for a minimum of 3 h. The media was changed to either fresh serum free media, serum free media with 100 ng/ml recombinant active two-chain HGF/SF (R&D Systems, Minneapolis, MN) or pro-HGF/SF with or without either 2 µM Aprotinin (MP Biomedicals, LLC, Solon, OH), 20 µM Leupeptin (Fisher Bioreagents, Fair Lawn, New Jersey), 60 nM HAI-1, 40 nM HAI-2 (R&D Systems), or 1 µM IN-1<sup>33</sup>. Inhibitors were added concomitantly with either pro-HGF/SF or HGF/SF, with the exception of IN-1, where the cells were pre-incubated for 30 min prior to growth factor stimulation.

### **3D cell culture**

Cells were plated on Matrigel Matrix Growth Factor Reduced (MMGFR)(BD Biosciences, Franklin Lakes, NJ) with serum free Mammary Epithelial Cell Growth Medium (MEGM<sup>TM</sup>) (Lonza, Walkersville, MD) containing the BulletKit<sup>TM</sup> growth supplement (BPE, hydrocortisone, GA-1000, Insulin). An overlay of MEBM growth media containing 2% MMGFR was added 1 hour after seeding<sup>56</sup>. Spheroids were allowed to form overnight before addition of 100 ng recombinant HGF/SF (R&D Biosystems, Minneapolis, MN) or



pro-HGF conditioned media. After treatment, cells were stained with 5  $\mu$ M Cell Tracker Orange (Invitrogen, Carlsbad, CA) for 45 min and Hoechst 33342 (Life Technologies Carlsbad, CA) for 5 min prior to imaging. Confocal images were acquired on a Zeiss LSM 780 scope at the Microscopy Imaging and Cytometry Resources Core at Wayne State University School of Medicine. Light microscopy pictures were acquired using a Nikon TMS microscope courtesy of Dr. Hyeong-Reh Kim (Wayne State University, Department of Pathology). RNAi silencing: For matriptase knockdown in SUM229, BT20 AND HCC1937 breast cancer cells, three independent Stealth RNAi<sup>TM</sup> siRNA duplexes (Invitrogen, Carlsbad, CA) targeting matriptase (siM1=ST14HSS186125, siM2=ST14HSS186126 siM3=ST14HSS110268) were used<sup>5</sup>. Two independent Stealth RNAi<sup>TM</sup> siRNA duplexes targeting uPA (siuPA1=PLAUHSS10878, siuPA2=PLAUHSS10876). Matched %GC negative controls (12935-300 and 12935-400) were used. Cells were transfected using Lipofectamine<sup>®</sup> RNAiMAX (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Two days after transfection, the cells were serum starved and used in the pro-HGF/HGF activation assays, in the 3D invasion assays, and in the proliferation assays.

### Transient transfection of SUM159 cells

SUM159 cells growing in 6-well plates were transfected when they reached 85% confluency using Lipofectamine LTX (Invitrogen, Carlsbad, CA) according to manufacturer's protocol. The DNA construct used was the mammalian expression vector, pcDNA3.1 containing full length human matriptase cDNA (a generous gift from Dr. Stine Friis, NIH)<sup>57</sup>.

### Western blot analysis

Mouse tissues were snap frozen and homogenized in ice cold RIPA lysis buffer (150 mM NaCl, 50 mM Tris, 1% NP-40, 0.1% SDS, pH 7.4) with protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) and phosphatase inhibitor (Sodium Orthovanadate, Sigma-Aldrich, St. Louis, MO). Cultured mouse and human cells were lysed in ice cold RIPA buffer with protease inhibitor cocktail and phosphatase inhibitor. Protein concentrations were determined by BCA assay (Pierce, Rockford, IL) and lysates were separated by 4–12% reducing SDS-PAGE and blotted onto PVDF membranes (Invitrogen, Carlsbad, CA). The following primary antibodies were used for detection: sheep anti-matriptase (1:500) (R&D Systems, Minneapolis, MN), rabbit anti-matriptase (1:1000) (Calbiochem/EMD Millipore, San Diego, CA), rabbit anti-phospho cMet (1:500), anti-phospho Gab1 (1:1000), anti-Gab1 (1:1000 dilution), anti-phospho Akt (1:1000), anti-Pan Akt (1:1000), mouse anti-human cMet (all from Cell Signaling Technology, Beverly, MA), rabbit anti-uPA (1:500) (Abcam, Cambridge, MA), and mouse anti-beta-actin (Sigma St. Louis, MO, 1:1000). Recombinant human pro-uPA was from American Diagnostica Inc. (Stamford, CT). For detection, secondary antibodies conjugated with either alkaline phosphatase (Sigma, St. Louis, MO) or horseradish peroxidase (Chemicon, Temecula, CA) in combination with either the chromogenic substrate nitro-blue tetrazolium and 5-bromo-4-chloro-3'-indolylphosphate solutions (Roche, Indianapolis, IN)(for analysis of human breast cancer cell and primary mammary carcinoma cell extracts) or Super-Signal West Femto Chemiluminescent Substrate (Pierce, Rockford, IL)(for analysis of whole tissue extracts of mammary glands and mammary tumors) were used. Examples of non-cropped scans of western blot membranes are shown in Supplementary Figs. 9–11.

### Proliferation assays in human breast cancer cell lines

30,000 human breast cancer cells were seeded in a six-well plates and serum starved overnight before stimulation with 100 ng/ml active HGF or pro-HGF for 24 h. Thereafter, cells were trypsinized and recounted using a hemocytometer. In the matriptase inhibition assay using IN-1, the synthetic inhibitor was added concomitantly with active HGF or pro-HGF.

### Matriptase gelatin zymography

Serum-free medium conditioned for 4 d by mouse mammary primary cells was collected, centrifuged to remove cell debris, and dialyzed against distilled water overnight at 4°C<sup>46</sup>. The dialyzed samples were lyophilized, and the dried protein powder was dissolved in a 1/100 volume of the initial conditioned medium in 20 mM Tris-HCl (pH 7.5). Concentrated medium was separated by SDS-PAGE under nonreducing conditions, and the gels (containing 0.1% gelatin) were subsequently incubated in renaturation buffer (50 mM Tris-HCl at pH 7.5, 100 mM NaCl, and 2.5% Triton X-100) for 1.5 h followed by 30 min in EDTA buffer (50 mM Tris-HCl at pH 7.5, 0.5 mM EDTA) to eliminate matrix metalloproteinase. The gels were incubated at 37°C for 16 h in developing buffer (50 mM Tris-HCl at pH 7.5 with 5 mM CaCl<sub>2</sub>), and stained with Coomassie Brilliant Blue to detect zones of gelatinolysis.

### Statistical analysis

All analysis was performed and graphs generated using Prism 5 software (GraphPad Software Inc., San Diego, CA). For comparisons between two groups student's t test was used. Non-parametric data was compared using the Mann-Whitney test. For correlation data, two estimators of association were used. The tetrachoric correlation coefficient assumes that the variables, matriptase and c-Met, in this case, are normally distributed but that they have been dichotomized. Kendall's tau does not have the assumption of normality and is a measure of concordance. Both measures have a theoretical range from -1 to 1.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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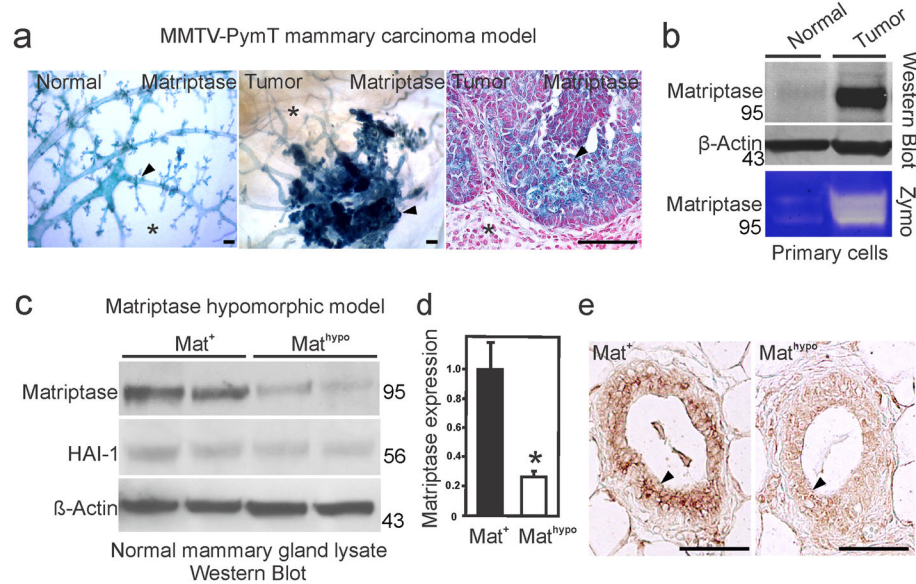
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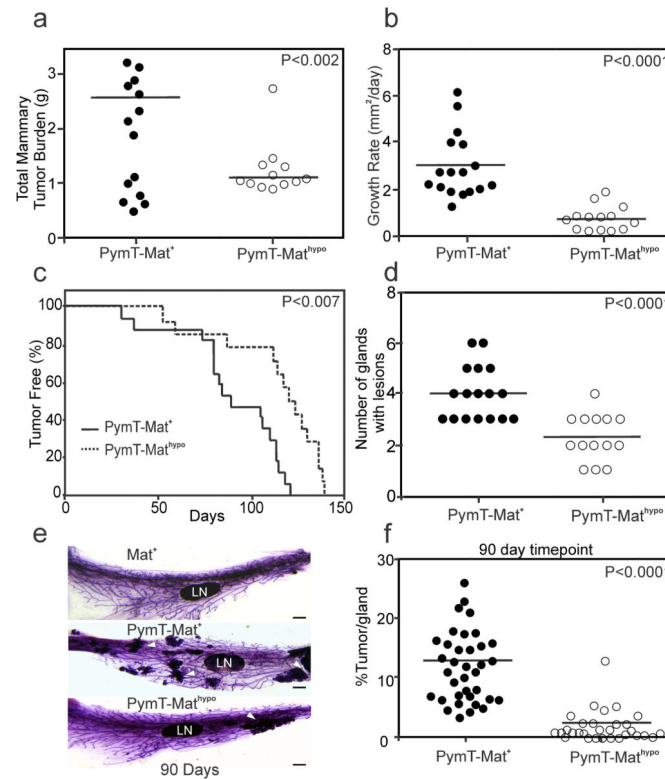




**Figure 1. Expression of matriptase in the MMTV-PymT mammary carcinoma model and matriptase hypomorphic model**

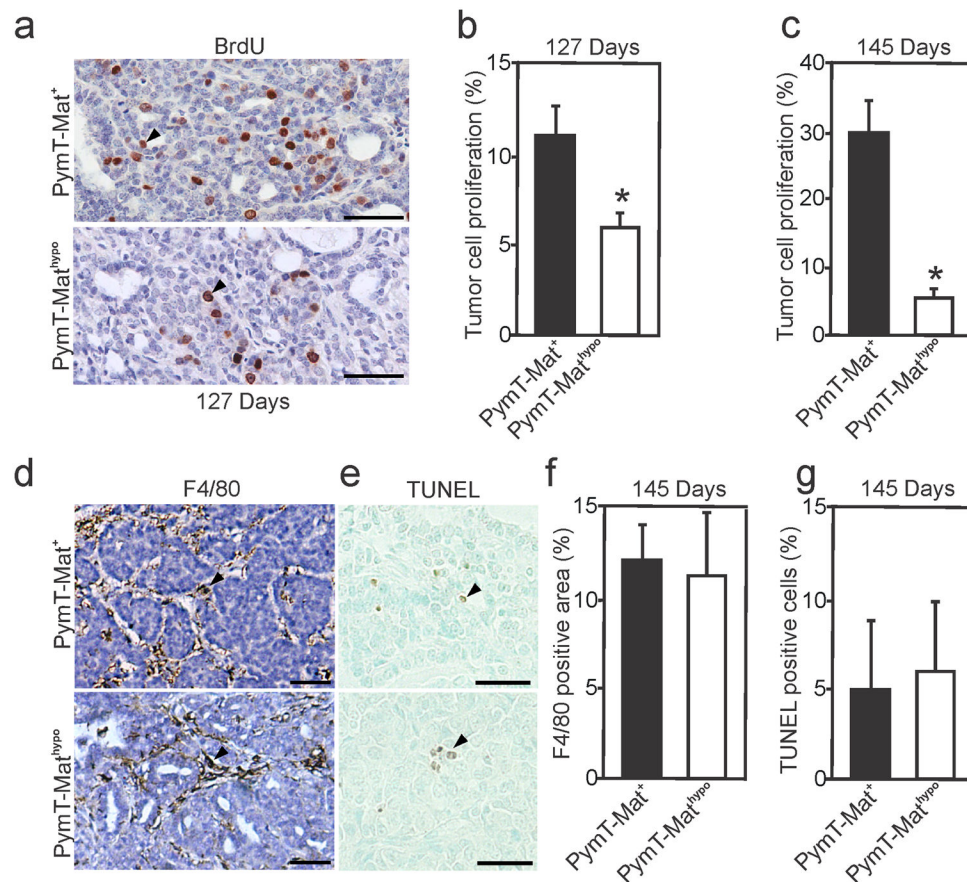
(a) A knock-in mouse with a promoterless  $\beta$ -galactosidase marker gene inserted into the endogenous matriptase gene was used for precise assessment of endogenous matriptase expression in the mammary gland by X-gal staining (blue color). Matriptase is expressed in the mammary gland epithelium (arrowhead in a) with no detectable expression in the stroma (\*) in whole mounts from normal glands (left panel) and MMTV-PymT induced carcinomas (middle panel). Histological analysis shows matriptase expression in the mammary cancer cells of MMTV-PymT transgenic mice (arrowhead, right panel) (b) Mammary epithelial cells from normal glands and tumor bearing glands were isolated and analyzed by western blotting (top panel) and gelatin zymography (lower panel) demonstrating increased levels of active matriptase in mammary carcinoma cells. (c) Western blot analysis of whole mammary gland extracts from matriptase hypomorphic mice (Mat<sup>hypo</sup>) and control (Mat<sup>+</sup>) littermates with anti-matriptase (upper panel) or anti-HAI-1 (middle panel) antibodies. The levels of matriptase are greatly reduced in the Mat<sup>hypo</sup> mice. Importantly, levels of the epithelia-specific, physiological matriptase inhibitor, HAI-1, are comparable in Mat<sup>hypo</sup> and Mat<sup>+</sup> glands. (d) Relative matriptase expression in Mat<sup>hypo</sup> mice (N=3) and Mat<sup>+</sup> mice (N=3) was determined by densitometry. Matriptase levels were normalized to HAI-1. Error bars represent S.D.  $P < 0.04$ , Student's t-test. (e) Immunohistochemical staining of mammary glands from virgin females with a sheep anti-matriptase antibody confirming that matriptase protein expression in the ductal epithelium is greatly reduced in the Mat<sup>hypo</sup> mice. Scale bars = 50  $\mu$ m.





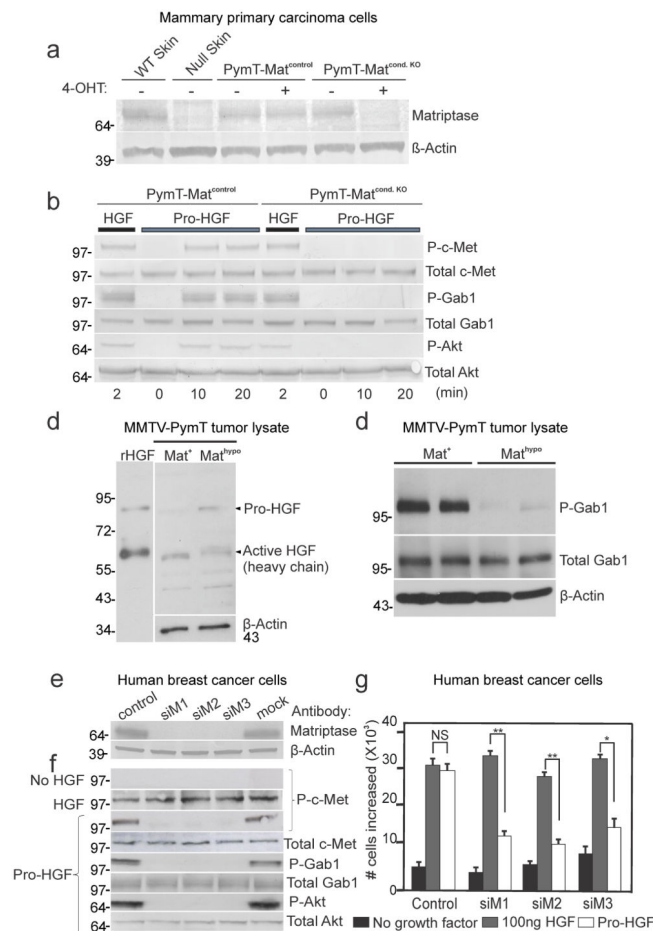
**Figure 2. Reduced matriptase decreases mammary tumor burden, growth rate, latency, and multiplicity**

A prospective cohort of littermate PymT- Mat<sup>+</sup> mice (N=14) and PymT Mat<sup>hypo</sup> mice (N=12) was followed over time. **(a)** Tumor burden at 145 days. Mice were euthanized and their mammary tumors resected and the total tumor weight was recorded for PymT Mat<sup>+</sup> mice (closed circles) and PymT-Mat<sup>hypo</sup> mice (open circles). Mean values were 2.6 and 1.1, respectively ( $P < 0.002$ ). **(b)** Mammary tumor growth rate for PymT-Mat<sup>+</sup> mice (closed circles) and PymT-Mat<sup>hypo</sup> mice (open circles). Mean values were 2.9 and 0.7, respectively ( $P < 0.0001$ ). **(c)** Kaplan-Meier tumor-free survival curves of PymT-Mat<sup>+</sup> (black line) and PymT-Mat<sup>hypo</sup> (dotted line). The median tumor-free survival times were 89 days versus 122 days, respectively ( $P < 0.007$ ), Chi-Squared Test. **(d)** Total number of glands with lesions at 145 days for PymT- Mat<sup>+</sup> mice (closed circles) and PymT-Mat<sup>hypo</sup> mice (open circles). Mean values were 4.0 and 2.2, respectively ( $P < 0.0001$ ). **(e)** Representative whole mounts of inguinal mammary glands from 90 day old virgin female littermates. Normal mammary epithelium in Mat<sup>+</sup> gland (upper panel), extensive growth of multiple lesions (arrowheads) in PymT-Mat<sup>+</sup> gland (middle panel) and one small localized lesion (arrowhead) in PymT-Mat<sup>hypo</sup> gland (lower panel). LN=Lymph node. **(f)** Total area of lesions in inguinal mammary glands from PymT-Mat<sup>+</sup> (closed circles) and PymT- Mat<sup>hypo</sup> (open circles) ( $P < 0.0001$ ). Mann-Whitney U Test was used in a, b, d, and f. Scale bars=1 mm.



**Figure 3. Decreased carcinoma cell proliferation in mammary tumors with reduced matriptase**

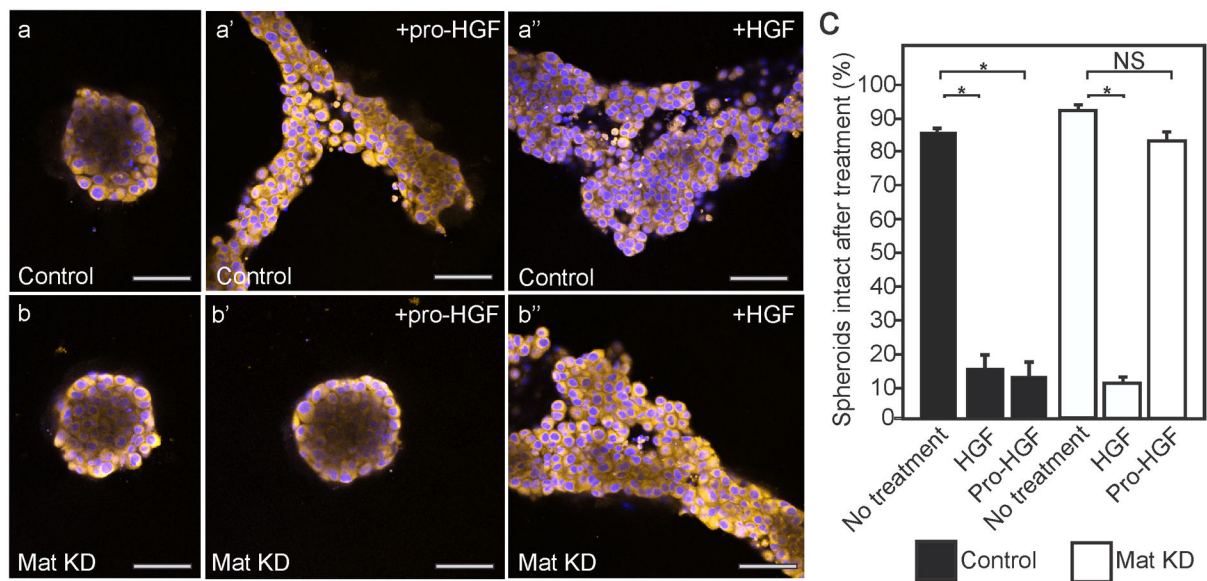
(a) Representative pictures of mammary tumors from PymT-Mat<sup>+</sup> (top) and PymT-Mat<sup>hypo</sup> (bottom) stained with an anti-BrdU antibody. Mice were injected with BrdU 2 h prior to euthanasia. (b) Quantification of carcinoma cell proliferation in 127 day old mice. Data are presented as the mean relative number of cells with positive nuclear BrdU staining (arrowheads) in tumors from PymT-Mat<sup>+</sup> (N = 4, black bar), and littermate PymT-Mat<sup>hypo</sup> mice (N = 3, white bar). (c) Quantification of carcinoma cell proliferation in 145 day old mice. Data are presented as the mean relative number of cells with positive nuclear Ki67 staining in tumors from PymT-Mat<sup>+</sup> (N = 4, black bar), and littermate PymT-Mat<sup>hypo</sup> mice (N = 4, white bar). (d) Tumor-associated macrophages in 145 day old mice. Macrophages were visualized using a rabbit anti-F4/80 antibody (arrowheads). (e) Apoptotic carcinoma cells detected by TUNEL staining in 145 day old mice (arrowheads). (f) Quantification of tumor-associated macrophages. Data are presented as the mean relative area with positive F4/80 staining in tumors from PymT-Mat<sup>+</sup> (N = 4, black bar), and littermate PymT-Mat<sup>hypo</sup> mice (N = 3, white bar). (g) Quantification of cells with TUNEL positive nuclear staining in tumors from PymT-Mat<sup>+</sup> (N = 4, black bars), and littermate PymT-Mat<sup>hypo</sup> mice (N = 3, white bars). All panels: Error bars represent S.D., \*P < 0.001, Mann-Whitney U Test. Scale bars = 50 μm.



**Figure 4. Matriptase is required for activation of the pro-HGF/c-Met signaling pathway and cell proliferation in primary mammary carcinoma cells, mammary tumors, and human breast cancer cells**

(a) Efficient Cre-mediated ablation of matriptase in primary mammary carcinoma cells shown by western blot analysis of cells isolated from PymT-Mat<sup>cond</sup> KO or PymT-Mat<sup>control</sup> mice. Two days after plating, 10  $\mu$ M 4-OHT (+) or vehicle (-) was added 24 h to ablate matriptase expression. Cells were in serum-free medium without 4-OHT for another 24 h before lysis. Skin lysates from newborn matriptase null and wild-type littermate mouse were included for antibody specificity. Anti-beta-actin was used as control for equal loading. (b) Mammary carcinoma cells were isolated and treated with 4-OHT as above and exposed to active HGF (2 mins) or pro-HGF (0, 10, or 20 mins). Cell lysates were analyzed for total c-Met, activated c-Met, and its downstream targets by western blotting. (c-d) Whole lysates of mammary tumors were separated by reducing SDS-PAGE and analyzed by western blotting. (e) HGF is mainly present in its active two-chain form in PymT-Mat<sup>+</sup> tumors, whereas a significant level of single-chain, inactive pro-HGF is detected in PymT-Mat<sup>hypo</sup> tumors. rHGF=recombinant pro-HGF/HGF. (d) reduced Gab1 levels of the active phosphorylated form relative to total Gab1 in PymT-Mat<sup>hypo</sup> mammary tumors (e) Efficient RNAi silencing of matriptase in human HCC1937 cells with three non-overlapping synthetic RNA duplexes (siM1, siM2, and siM3). A %GC matched RNA duplex was used as negative control as well as a mock (no RNA duplex). Beta-actin staining ensured equal protein loading. (f) Cells

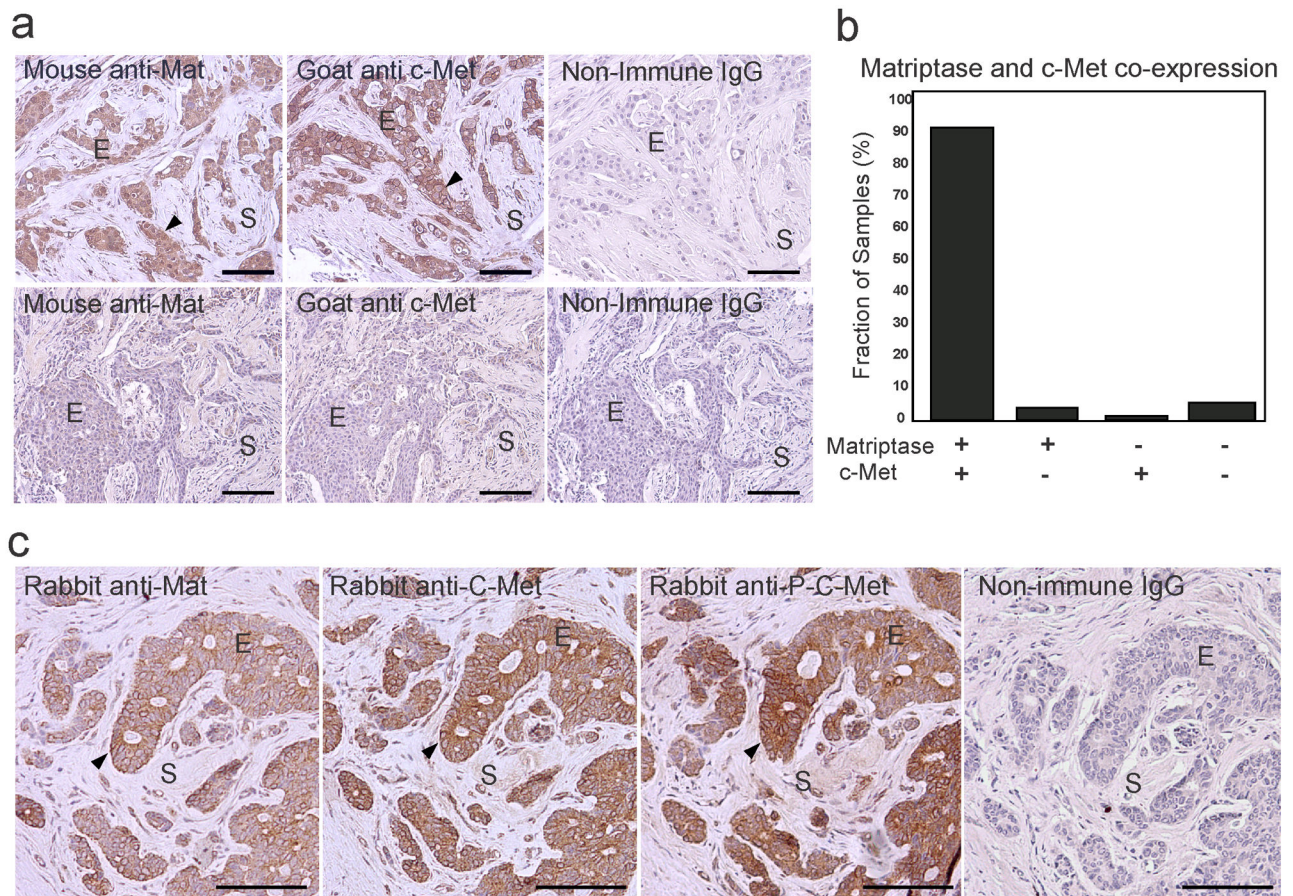
were serum starved and exposed to no HGF, active HGF (2 min), or pro-HGF (15 min). Lysate were analyzed for total c-Met, activated c-Met, and its downstream targets. **(g)** Matrilysin was silenced in HCC1937 cells as above and left untreated (black bars) or exposed to active HGF (gray bars) or pro-HGF (white bars) for 24 h. Bar graphs show proliferation 24 h after stimulation (\* $P < 0.04$ , \*\* $P < 0.005$ , \*\*\* $P < 0.0008$ , Student's t-test). Data represent mean of three replicates. Error bars represent S.D. Data are representative of four independent experiments.



**Figure 5. Matriptase mediates pro-HGF induced invasion in human breast cancer cells**

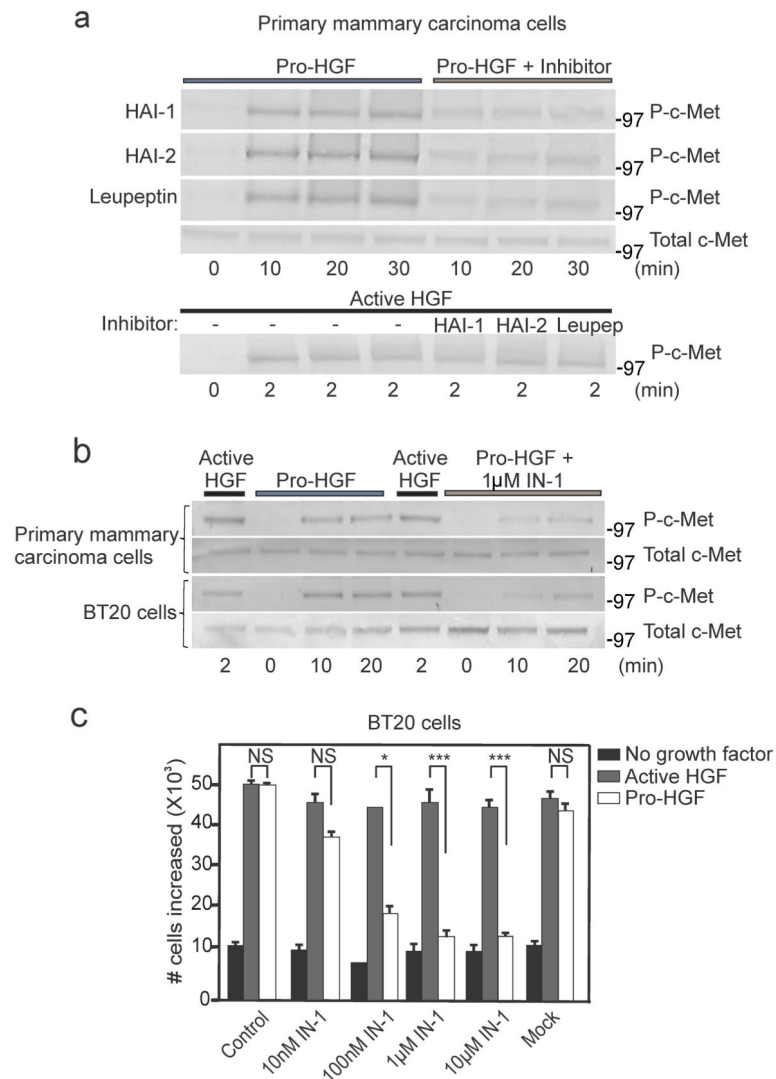
SUM229 cells were grown on culture plates and matriptase was silenced (Mat KD) using synthetic RNA duplexes (B, B', B''). A %GC matched RNA duplex was used as negative control (a, a', a''). Cells were allowed to form spheroids in 3D rBM overlay culture for 24 h before addition of either pro-HGF (a', b'), HGF (a'', b'') or no growth factors (a, b) and imaged by confocal microscopy. When no growth factors were added, control and Mat KD spheroids remained intact (black arrowheads). In contrast, addition of pro-HGF induced extensive invasive outgrowths in matriptase sufficient control cells (a') (indicated with open arrowheads) whereas the majority of Mat KD spheroids remained intact (b'). Both control and Mat KD spheroids responded to active two-chain HGF (b''', b'''). Cells were labeled with Cell Tracker Orange and nuclei with Hoechst (blue) (c) 3D rBM cell cultures were scored for the number of intact spheroids when left untreated (black bars) or exposed to either active HGF (white bars) or pro-HGF (white bars). Data represent mean of the number of intact spheroids before treatment relative to the number after treatment (triplicates) (\*P < 0.04, Student's t-test). Error bars represent S.D. Scale bars=50  $\mu$ m. Data are representative of four independent experiments.





**Figure 6. Matriptase and c-Met expression is correlated in human invasive ductal carcinomas**

Representative staining of serial sections of invasive ductal carcinoma **(a)** using a mouse monoclonal anti-matriptase antibody and a goat anti-c-Met antibody. Upper panels: carcinoma with matriptase and c-Met co-expression. Lower panels: carcinoma negative for both matriptase and c-Met. Primary antibodies were substituted with non-immune control IgG as negative controls. **(b)** Expression of matriptase and c-Met in 153 human invasive ductal carcinomas. Bars depict the frequency of samples expressing matriptase and c-Met (134/153), samples that express neither matriptase nor c-Met (11/153), samples that express only matriptase (7/153), and samples that express c-Met only as indicated (1/153). The overall comparison of groups showed a highly significant correlation between c-Met and matriptase expression: Tetrachoric Rho = 0.95 ( $P < 0.001$ ). **(c)** Representative staining of serial sections of invasive ductal carcinoma using rabbit anti-matriptase, rabbit anti-c-Met, and rabbit anti-phospho-c-Met. Matriptase, c-Met, and phospho-c-Met (brown staining, arrow heads) is detected in the epithelia-derived cancer cells (indicated with “E”) with no significant staining in the mesenchymal/stromal compartments (indicated with “S”). Tissues were counterstained with hematoxylin (blue/grey). Both matriptase and c-Met are primarily localized on cell surfaces and in the cytoplasm and display highly similar expression patterns. Scale bars = 100  $\mu$ m.



**Figure 7. Abrogation of pro-HGF mediated c-met activation and breast cancer cell proliferation using matriptase inhibitors**

**(a)** Matriptase sufficient mouse mammary carcinoma cells were isolated from Mat-PymT<sup>+</sup> mice and exposed to pro-HGF or pre-cleaved active HGF with and without matriptase inhibitors added concomitantly (60 nM HAI-1, 40 nM HAI-2 or 20 μM leupeptin). Cells were lysed at the indicated time points after growth factor stimulation and analyzed for total and activated c-Met by western blotting. **(b)** Mouse mammary carcinoma cells or human breast cancer BT20 cells were serum starved and pre-incubated with 1 μM IN-1 synthetic matriptase inhibitor for 30 min before exposure to active HGF, pro-HGF, or no growth factors. Cells were lysed at the indicated time points after growth factor stimulation and analyzed for total and activated c-Met by western blotting. **(c)** BT20 cells were serum starved and exposed to either active HGF (gray bars), pro-HGF (white bars), or no growth factor (black bars) for 24 h and counted. The synthetic selective matriptase inhibitor IN-1 was added at 10 nM, 100 nM, 1 μM, or 10 μM concomitantly with pro-HGF/HGF. In the mock control vehicle (PBS) was added. Graphs show the increase in cell numbers at 24 h



after stimulation (triplicates) (\* $P < 0.04$ , \*\* $P < 0.005$ , \*\*\* $P < 0.0008$ , Student's t-test). Error bars represent S.D. All data are representative of three independent experiments.

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